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Effects of collection methods and storage on the in vitro stability of canine plasma catecholamines

Michele M. D'Alesandro, PhD; Dale F. Gruber, PhD; H. Lester Reed, MD; Kevin P. O'Halloran; Robert Robertson

SUMMARY

Norepinephrine (NE) and epinephrine (EPI) collected from dogs were sequentially and temporally measured in blood and plasma at 24 C. Heparin and EDTA anticoagulants, in combination with reduced glutathione and EDTA as a preservative, were also compared. Norepinephrine and EPI concentrations were measured by high-pressure liquid chromatography with electrochemical detection. In heparinized plasma, NE and EPI concentrations were relatively stable in the absence or presence of preservative after 24 hours at 24 C. In EDTA plasma, NE and EPI values were less stable when compared with those in heparinized samples. Norepinephrine concentrations in EDTA plasma without preservative decreased by 163.2 ± 8.88 pg over 24 hours, compared with an 86.6 ± 7.92 pg loss of NE in heparinized plasma. The degradation of EPI in EDTA plasma without preservative was also twofold greater, compared with that in heparinized plasma. Addition of preservative had no stabilizing effect on NE or EPI in heparinized or EDTA plasma. During long-term storage at −70 C, plasma NE and EPI values decreased < 0.6 and < 0.1 pg/d, respectively. Norepinephrine and EPI values were stable in heparinized blood for 6 hours but decreased to < 25% and < 6% of initial base line values, respectively, when plasma separation was delayed 24 hours.

Concentration of canine plasma norepinephrine (NE) and epinephrine (EPI) has been used to assess the alteration(s) of metabolic and hemodynamic responses to induced hypotension[1] and lactic acidosis as a result of tissue hypoxia.[2,3] Severe metabolic acidosis experienced by dogs has been attributed to increased sympathetic activity, as evidenced by high plasma NE values. Because much research in critical care medicine is directed toward patient resuscitation after shock and/or trauma, the relevance of changes in plasma catecholamine content is evident.

The stability of catecholamines in plasma and blood must be maintained because the chemical structure of the catechol ring is susceptible to spontaneous oxidation. Quantitative analyses of catecholamines have included fluorometric techniques,[4] radioenzymatic assays,[5,6] and high-pressure liquid chromatography (HPLC).[7,8] High-pressure liquid chromatography with electrochemical detection has the least interlaboratory variability, compared with other analytic techniques.[9] In conjunction with reports of quantitation by various techniques, differences in the method of blood collection, choice of anticoagulant, type and addition of antioxidant, and sample processing procedures have also been reported.[8,10,11]

The purpose of the study reported here was to use HPLC to measure NE and EPI degradation in plasma stored at −70 C and at 24 C from heparinized or EDTA plasma in the presence or absence of reduced glutathione (GSH) and EGTA. In addition, NE and EPI degradation in blood at 24 C was determined.

Materials and Methods

Dogs—Twelve purpose-bred healthy male Beagles (1 to 2 years old, 10 to 12 kg) were offered commercial dog food and tap water ad libitum. Animal holding rooms were maintained at 20 C with 50 ± 10% relative humidity, using at least 10 air changes of 100% conditioned fresh air/h. Dogs were maintained on a 12-hour lighting cycle with no twilight.

Sample collection—Blood was collected by venipuncture from the lateral saphenous vein into syringes containing EDTA (4 mmol/L) or heparin (14.3 USP U/ml). All blood samples, except those to be used for blood degradation studies were centrifuged within 1 hour of collection.

Blood samples were centrifuged at 1,000 × g for 10 minutes at 4 C to pellet cellular elements and platelets. The plasma supernatant was aliquoted (1 ml) into 1.5-ml microcentrifuge tubes with or without EGTA (8 mmol/L, final concentration) and GSH (6.5 mmol/L, final concentration) as a preservative. Samples were frozen immediately at −70 C as controls and for analysis after long-term storage or were incubated at room temperature (24 C) for 0, 0.5, 1, 2, 3, 4, 10, and 24 hours before storage at −70 C. Degradation of plasma NE and EPI in heparinized blood was analyzed after gentle rotation at 24 C for 0, 0.5, 4, 8, 24, 48, and 72 hours before removal of plasma and storage at −70 C. Plasma used for 24 C degradation analysis and for analysis after long-term storage was pooled from a minimum of 6 dogs. Plasma pools were different for various experiments. The inherent individual variability of NE and EPI values observed in dogs accounted for differences in the initial baseline values of these pooled samples. For blood studies, sufficient blood was obtained and analyzed individually from 4 dogs to avoid pooling of blood.

Catecholamine extraction and quantitation—Plasma samples were extracted as described.[12] Alumina adsorption of catecholamines was complete after 30 minutes at 24 C. After the adsorbed alumina was washed, catecholamines were released by addition of 100 μl of acetic acid containing 0.05% EDTA and 0.1% sodium disulfite. To determine extraction efficiency and quantitate catecholamine values, 3,4-dihydroxybenzylamine was used as an internal standard. Samples were assayed by HPLC with

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electrochemical detection[a] and were computer analyzed.[b] Extraction and HPLC methods allow for reliable detection of NE and EPI concentrations > 20 pg/ml. The intraassay coefficient of variation for NE was 5% and for EPI was 8%. Assays were run in duplicate, except for the long-term degradation samples that were run in triplicate. The conversion factor for expressing NE as nmol/L is 0.00591 and for expressing EPI as pmol/L is 5.458. Statistical differences were determined by analysis of variance with the Duncan test between means for repeated measures.[c]

Results

Plasma catecholamine degradation—Plasma NE values from heparinized blood decreased after 24 hours at 24 C, from 615 ± 4.5 pg/ml to 531 ± 3.0 pg/ml (without GSH/EGTA) and 523 ± 3.0 pg/ml (with GSH/EGTA; Fig 1), respectively. Decay rates of 86.64 ± 7.92 and 74.88 ± 12.48 pg/d, respectively, were not significantly different (Table 1). Without preservative, EPI values in heparinized plasma decreased in 24 hours from 407 ± 5 to 350 ± 3 pg/ml, with a decay rate of 45.12 ± 10.80 pg/24 h (Table 1). With preservative added, EPI values in heparinized plasma decreased from 393 ± 7 to 372 ± 10 pg/ml, with a significantly slower decay rate of 24.96 ± 5.04 pg/24 h.

Norepinephrine and EPI values in EDTA plasma were less stable, compared with those in heparinized plasma (Fig 2). Norepinephrine values decreased from 411 ± 7 to 274 ± 12 pg/ml (decay rate, 163.2 ± 8.88 pg/24 h) without preservative and from 407 ± 5 to 258 ± 18 pg/ml (decay rate, 143.3 ± 9.84 pg/24 h) with preservative added. Epinephrine values decreased from 411 ± 19 to 303 ± 8 pg/ml (decay rate, 139.68 ± 15.84 pg/24 h) without preservative and from 443 ± 14 to 282 ± 29 pg/ml (decay rate, 159.84 ± 18.84 pg/24 h) with preservative added. The NE and EPI decay rates in EDTA plasma were significantly greater than those observed in heparinized plasma. In addition, EPI was most stable when anticoagulated with heparin and stored in the presence of GSH/EGTA.

Long-term catecholamine stability—After long-term storage at −70 C, heparinized samples containing preservative were quantitatively analyzed to measure NE and EPI degradation (Table 2). The mean decay rate for NE was 0.5 ± 0.2 pg/d and 0.1 ± 0.05 pg/d for EPI after 116 days' storage.

Degradation of catecholamines in blood—Norepinephrine values decreased to < 25% and EPI to < 6% of initial

[a] Waters 460 Electrochemical Detector, Waters Millipore Corp, Bedford, Mass.
[b] Waters Expert Chromatography Software (version 5.2), Waters Millipore Corp, Bedford, Mass.
[c] STATPAK (version 4.1), Northwest Analytical Inc, Portland, Ore.

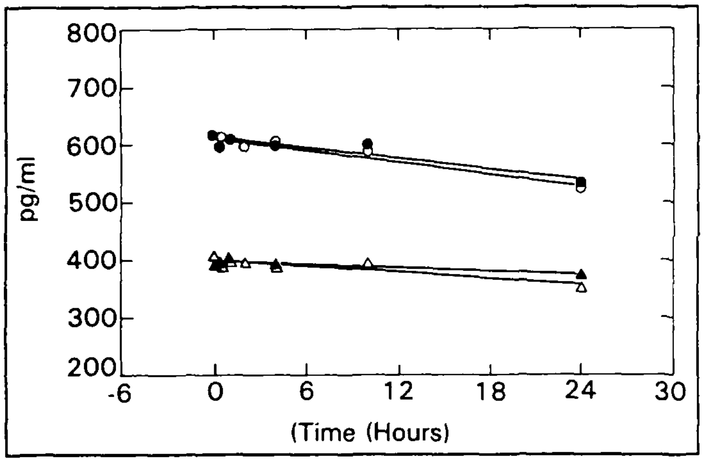

Fig 1—Changes in canine norepinephrine (NE) and epinephrine (EPI) values in heparinized plasma. Plasma was incubated at 24 C for indicated times in the presence or absence of reduced glutathione (GSH)/EGTA; ●——● = NE without GSH/EGTA; ○——○ = NE with GSH/EGTA; ▲——▲ = EPI without GSH/EGTA; △——△ = EPI with GSH/EGTA.

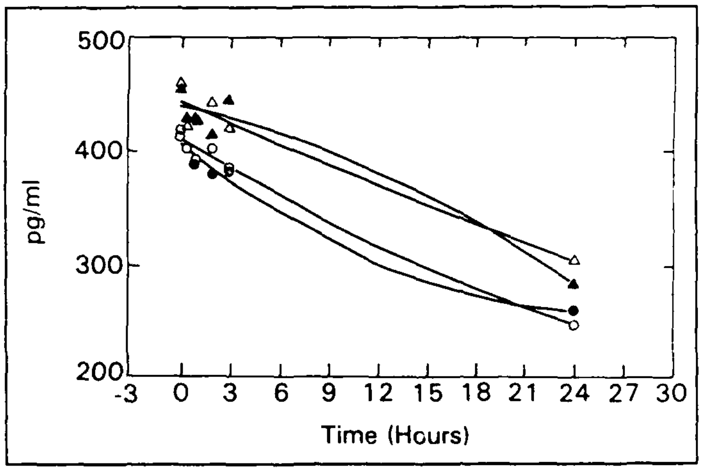

Fig 2—Changes in NE and EPI values in EDTA plasma. Plasma was incubated at 24 C in the presence or absence of GSH/EGTA; ●——● = NE without GSH/EGTA; ○——○ = NE with GSH/EGTA; ▲——▲ = EPI without GSH/EGTA; △——△ = EPI with GSH/EGTA.

TABLE 1—Quantitative changes in norepinephrine (NE) and epinephrine (EPI) in pooled samples of heparinized or EDTA plasma (from ≥ 6 dogs) in the absence (−) or presence (+) of reduced glutathione (GSH) and EGTA

	GSH/EGTA	Control (pg/ml)	Y-intercept (pg/ml)	Decay rate (pg/24 h ± SEM)	n
Heparin					
NE	−	615 ± 5	612	86.6 ± 7.92	7
	+	616 ± 4	613	74.9 ± 12.48	7
EPI	−	407 ± 5	410	45.1 ± 10.80*	7
	+	393 ± 7	399	25.0 ± 5.04	7
EDTA					
NE	−	411 ± 7	409	163.2 ± 8.88†	6
	+	407 ± 5	400	143.3 ± 9.84†	6
EPI	−	441 ± 19	442	139.7 ± 15.84*	6
	+	443 ± 14	443	159.8 ± 18.84*	6

* $P < 0.05$, compared with EPI values from heparinized plasma with GSH/EGTA. † $P < 0.05$, compared with NE values from heparinized plasma with GSH/EGTA.
Control assays were run in duplicate. Decay rates were determined by linear regression analysis. n = No. of dogs.

TABLE 2—Changes in the mean concentration (± SEM) of plasma NE and EPI during long-term storage at −70 C

Day	NE (pg/ml)	EPI (pg/ml)
1	486 ± 13	257 ± 7
40	431 ± 23	244 ± 14
60	446 ± 16	250 ± 12
75	437 ± 39	246 ± 40
116	415 ± 12	244 ± 5
Decay rate (pg/d ± SEM)	0.5 ± 0.2	0.1 ± 0.05

Samples were pooled heparinized plasma from a minimum of 6 dogs. Decay rates were determined by linear regression analysis. Assays were run in triplicate.
See Table 1 for key to abbreviations.

TABLE 3—Quantitative changes in plasma NE and EPI during delayed plasma separation from heparinized blood

Time (h)	NE (pg/ml)	EPI (pg/ml)
0	388	515
4	348	247
8	321	155
24	88	28
48	43	< 20*
72	37	< 20*
Slope (pg/h)†	−12.75 ± 1.03	−16.88 ± 7.07
Decay rate (pg/24 h)†	−306 ± 24	−405 ± 170

* Below the level of detection with high-pressure liquid chromatography. † Data are expressed as mean ± SEM.

Decay rates were determined by linear regression analysis for the initial 24 hours at 24 C. Because of the individual variability of dogs, representative data are presented.

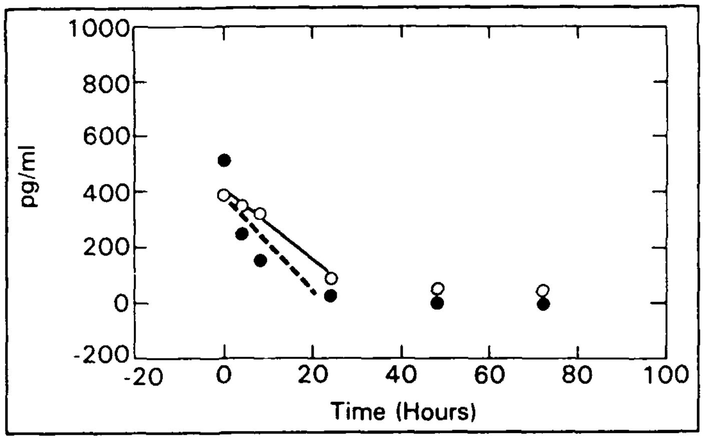

Fig 3—Degradation of NE (●—●) and EPI (O—O) in heparinized whole blood. Plasma was extracted and catecholamines were analyzed after gentle rotation of blood at 24 C for indicated times. Decay rates were determined for the initial 24 hours at 24 C by linear regression analysis.

baseline values in blood gently rotated for 24 hours at 24 C (Table 3). Norepinephrine decayed at a rate of 12.75 ± 1.03 pg/h, and EPI decayed by 16.88 ± 7.07 pg/h. The time course appeared to be curved, yet linear estimations of the decay rates were computed for the initial 24 hours because there were insignificant changes after 48 hours (Fig 3).

Discussion

Methods for measuring plasma catecholamines have required rapid processing and storage to prevent the spontaneous degradation of the catechol ring. These requirements have led to logistic complications between clinics or field sites, and distant laboratories equipped for analysis. We measured degradation of NE and EPI values in plasma samples maintained at room temperature (24 C) for periods that are likely to take place when (i) samples are collected outside of normal operating hours of the laboratory, (ii) samples must be transported for analysis, or (iii) in the event of freezer malfunction. In the absence of antioxidants, degradation of NE and EPI did not take place, using heparin or EDTA as the anticoagulant for 6 hours. Epinephrine values were stable in heparinized plasma for 24 hours, and NE values decreased by < 15%. In EDTA plasma, there was significant degradation of NE and EPI values within 24 hours. These results are similar to those in studies on human beings that indicated catecholamines to be relatively stable for 3 hours in EDTA[10] and for up to 22 hours in heparinized plasma.[11]

Catecholamines from human beings are stable in blood collected in EDTA for 3 hours[10,11] and for 6 hours in heparinized or EDTA blood.[d] Canine blood degradation of NE and EPI was measured with heparin only. In contrast to the stability evidenced in blood from human beings, NE and EPI concentrations in dogs decayed by > 3%/h. Seemingly, estimations of in vivo concentrations of catecholamines require a minimal delay in the separation of cellular elements and plasma. However, final freezing of the heparinized plasma sample can be delayed for up to 6 hours without significant degradation.

The concentration of circulating catecholamines has been used as an indicator of the efficacy of resuscitation methods and mechanisms contributing to the development of shock and trauma.[1-3] The relevance of circulating catecholamines to a comprehensive analysis of critical care medicine requires simplified procedures for the collection of blood and preparation of plasma samples to minimize degradation. Samples collected in field settings or at locales distant to the laboratory can be analyzed accurately to generate values that are quantitatively comparable with those in vivo.

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